

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 07/03/2000			2. REPORT TYPE Final Report		3. DATES COVERED 1 June 1996 - 30 Sept 1999	
4. TITLE AND SUBTITLE Regulation of attachment by a marine bacterium: bioadhesion proteins				5a. CONTRACT NUMBER N00014-96-1-0596		
				5b. GRANT NUMBER		
				5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) David L. Kirchman				5d. PROJECT NUMBER		
				5e. TASK NUMBER		
				5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Graduate College of Marine Studies University of Delaware Lewes, DE 19958				8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Office of Naval Research 800 N. Quincy Street Arlington, VA 22217-5000				10. SPONSOR/MONITOR'S ACRONYM(S)		
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT It is well known that bacteria are the first colonizers of surfaces placed in seawater and that fouling by bacteria and other microbes can have both beneficial and deleterious effects. However, we know little about the mechanisms of how bacteria attach to inert surfaces in aquatic environments. A molecular approach was used to examine the properties of attachment proteins and to explore their role in the initial events of bacterial fouling. Results with mutants of a marine bacterium <i>Vibrio harveyi</i> suggest that membrane proteins can mediate bacterial attachment to a variety of surfaces.						
15. SUBJECT TERMS Bacteria, fouling, attachment, adhesions, mutations						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON	
a. REPORT unclass- fied	b. ABSTRACT unclass- fied	c. THIS PAGE unclass- fied	unclassified	5	David L. Kirchman	
					19b. TELEPHONE NUMBER (Include area code) (302) 645-4375	

2000 310 042

FINAL REPORT

GRANT#: N00014-96-1-0596

PRINCIPAL INVESTIGATOR: David L. Kirchman

INSTITUTION: University of Delaware

EMAIL: Kirchman@udel.edu

GRANT TITLE: Regulation of attachment by a marine bacterium: bioadhesion proteins

REPORTING PERIOD: 1 June 1996 - 30 September 1999

AWARD PERIOD: 1 June 1996 - 30 September 1999

OBJECTIVES: To determine the role of proteins in mediating attachment by the marine bacterium *Vibrio harveyi*.

APPROACH: We use molecular and biochemical approaches to examine the role of specific proteins in microbial attachment.

ACCOMPLISHMENTS: Previous work had demonstrated that several membrane proteins are potentially involved in attachment of *V. harveyi* to surfaces (Montgomery and Kirchman 1993; 1994). The general goal of this project was to identify some of these proteins at the molecular level. The initial surface used in attachment assays was chitin because of its oceanographic relevance and because we reasoned that marine bacteria would be adapted to common natural surfaces such as chitin. Subsequent work demonstrated that chitin-binding proteins may also be involved in adhesion to manufactured surfaces and surfaces covered with organic polymers more closely related to those used in modern anti-fouling applications.

To isolate and examine potential attachment proteins, clone libraries from *V. harveyi* were constructed and screened with an antibody against ChiA, a chitinase (enzyme that hydrolyzes chitin) from this bacterium found by previous work. Several clones from this library were required to deduce the complete sequence of ChiA. Sequence analysis suggested the presence of a non-catalytic chitin-binding domain in ChiA. The actual role of this domain was confirmed by constructing with molecular tools truncated versions of ChiA. Removal of the chitin-binding domain greatly reduced binding of the enzyme and chitin hydrolysis. We speculated that the chitin-binding domain determines the movement of ChiA along chitin-containing surfaces.

20000310 042

Sequence analysis of the chitin-binding domain provides some insights into the nature of surface-protein and potentially microbe-surface interactions. The chitin-binding domain was found in the sequence of several proteins from taxonomically diverse microbes. Prominent among the conserved residues were aromatic amino acids, especially tryptophan, which have been shown to be important in hydrophobic binding of glycanases to polysaccharides. We observed similarity among the binding domains of chitinases, cellulases, and xylanases. These observations have implications for understanding protein binding and evolution of proteins via the shuffling of domains among distantly related bacterial species.

Another clone (pPV2) from the *V. harveyi* clone library produces a 107 kDa protein with chitin-binding capacities. Originally, we could not observe any chitinase activity associated with the clone nor the 107 kDa protein, but zymograms revealed three chitinases in crude extracts of *E. coli* bearing pPV2. Although the clone is large enough (7 kb) to encode several proteins, sequence analysis indicated that the clone synthesizes a single *V. harveyi* protein which is hydrolyzed by *E. coli* proteases to three chitinases.

Analysis of pPV2, which was entirely sequenced, revealed several interesting features. The protein (ChiB) contains a chitin-binding domain with the same conserved tryptophan residues as in other binding domains, but overall there was little similarity (30%) with the chitin-binding domain in ChiA. Unlike ChiA, the chitin-binding domain of ChiB is near the N-terminus of the protein. In addition to a catalytic domain similar to other chitinases, ChiB also has a glycosyl hydrolase family 3 domain.

We wished to explore the role of the pPV2-encoded proteins in attachment by *V. harveyi* to surfaces by creating mutants in *V. harveyi* using insertion mutagenesis with a kanamycin resistance cassette added to the suicide vector pGP704. Successful integration of the modified gene was verified by Southern blot analysis. Unexpectedly, attachment by the insertion mutants was higher, not lower, than attachment by the wild type to a wide variety of surfaces. In addition to chitin, we examined attachment to glass, sulphonated polyurethane, and aminated polyurethane as a function of shear (flow) along the surfaces. We observed differences in attachment by the mutants and wild type as a function of both shear and surface material. One mutant (Mutant M), for example, attached to glass and aminated polyurethane at faster rates than another mutant (Mutant B) and the wild type, but Mutant M attached slower (depending on shear rates) than Mutant B and the wild type to sulphonated polyurethane.

We are currently trying to understand the molecular

basis for the observed differences in attachment and to use this information to explore how *V. harveyi* attaches to surfaces. The insertion mutations apparently are in a regulatory region because selected membrane proteins are more abundant in the mutants than in the wild type. It is important to note that overall surface hydrophobicity and polysaccharide production do not differ from the wild type. A few overexpressed membrane proteins in the mutants appear to be causing the different attachment behavior.

To identify the regulatory region apparently governing the production of the presumed attachment proteins, we are currently identifying the insertion sites of the transposon mutants. Clone libraries of the mutants were prepared and screened for kanamycin resistance because these clones would have the suicide vector inserted into the *V. harveyi* chromosome.

CONCLUSIONS: The mutation studies indicate that attachment proteins are subjected to a complex regulatory system probably linked to the production of various extracellular hydrolyases. Further work is necessary to understand the details of this regulation, but the results provide further support for the hypothesis that several proteins are involved in the attachment of *V. harveyi*.

SIGNIFICANCE: Few studies have considered how specific cellular components such as proteins may mediate attachment by non-pathogenic bacteria. This work adds to our understanding of interactions between surfaces and microbes at the molecular level.

Award Information:

Editor-in-chief, *Limnology and Oceanography* (1993-1998)
Editorial Board, *Biofouling* (1996-)
Editorial Board, *FEMS Microbiological Ecology* (1997-)
Editorial Board, *Aquatic Microbial Ecology* (1995-)
Editorial Advisory Board, John Wiley & Sons (1997-)
Invited Gordon conference speaker (1997)
ASM committee on global change (1999)
Session Chairman for AGU-ASLO meetings (1998, 1999)
Organizing Committee for European Marine Microbiology Symposium (1999-2000)
Kirchman was named the Maxwell P. and Mildred H. Harrington Professor of Marine Studies in 1998.

PUBLICATIONS AND ABSTRACTS

1. Ni Chadhain, S.M. and D.L. Kirchman. 1996. Knockout mutagenesis of a high molecular weight chitin-binding

protein mediating attachment by the marine bacterium *Vibrio harveyi*. ASM General Meeting, New Orleans, LA.

2. Kirchman, D.L. 1996. Microbial ferrous wheel. *Nature* 383:303-304.

3. Kirchman, D.L. 1997. Microbial breathing lessons. *Nature* 385:121-122.

4. Svitil, Amy L., S.M. Ni Chadhain, J.A. Moore, and D.L. Kirchman. 1997. Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. *Appl. Environ. Microbiol.* 63:408-413.

5. Svitil, A.L. and D.L. Kirchman. 1998. A chitin-binding domain in a marine bacterial chitinase and other microbial chitinases: implications for the evolution of β -1,4-glycanases. *Microbiology (U.K.)* 144: 1299-1308.

6. Kirchman, D.L. 1998. Review of "Scholarly Publishing" (R.P. Peek and G.B. Newby, ed). *Limnol. Oceanogr.* 43: 1017-1018.

7. Kirchman, D.L. 1999. Phytoplankton death in the sea. *Nature* 398: 293-294.

8. Kirchman, D.L. 1999. Review of "Bacterial Biogeochemistry" (T.Fenchel, G.M. King, and T.H. Blackburn). *Limnol. Oceanogr.* 44: 233-234.

9. Kirchman, D.L. In press (Editor). *Microbial Ecology of the Oceans*. Wiley-Interscience.

10. Ni Chadhain, S.M., J. Grapski, S.L. Cooper, and D.L. Kirchman. In prep. Effect of surface properties and shear on attachment by a marine bacterium.

11. Ni Chadhain, S.M. and D.L. Kirchman. In prep. Role of specific proteins in regulating attachment of a marine bacterium.

12. Ni Chadhain, S.M. and D.L. Kirchman. In prep. Characterization of a multi-functional chitinase from a marine bacterium.

13. Ni Chadhain, S.M. In prep. Characterization at the molecular level of attachment and chitin degradation by *Vibrio harveyi*. Ph.D. Dissertation, University of Delaware.